INVESTIGATION OF THE EFFECT OF RUTIN ON TYPE II DIABETES MELLITUS ASSOCIATED NEUROLOGICAL COMPLICATIONS

SYNOPSIS

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ABSTRACT

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by consistently and prolonged hyperglycemia. Diabetes is associated with neurological complications like neurodegeneration, cognitive decline, dementia and even disorders like Alzheimer's and Parkinson's. Previous research in our lab elucidated a high quantity of rutin in *Urtica dioica* leaf extract, which showed pronounced neuroprotection against diabetic complications. So, the rationale of this study was to investigate whether or not, rutin could be the principal neuroprotective component in *Urtica dioica* leaf extract against type 2 diabetes (T2DM) mellitus associated neurological complications. We aim to identify the role of insulin signaling in brain during T2DM and the effect of rutin treatment on it.

Our first objective was to study the neurological complications of chronic diabetes and the effect of rutin in it. Diabetes was induced using multiple low dose of streptozotocin (STZ). Diabetes was allowed to progress for 8 weeks during which animals were treated with 100 mg/kg rutin (po; od). STZ treated animals showed steady increase in the blood glucose levels and development of glucose intolerance. Diabetic animals were highly anxious, depressed and showed marked learning and memory dysfunction. These behavioral deficits were attributed to impaired hippocampal insulin signaling. Rutin treatment improved hyperglycemia, glucose intolerance and behavioral dysfunction by upregulating the hippocampal insulin signaling.

Our next objective was to develop an insulin resistant state in mice, as observed in T2DM. For this we employed a 21 day chronic unpredicted stress (CUS) induced depression paradigm, along with rutin treatment. CUS induced pre-diabetic state, insulin resistance (IR) and behavioral dysfunctions, which were associated with hippocampal neurodegeneration and impaired insulin signaling. 21 day rutin treatment (100 mg/kg; po; od) improved glucose homeostasis, insulin resistance, behavioral dysfunctions and alleviated hippocampal neurodegeneration. Mechanistically, rutin modulated the hippocampal insulin signaling pathway by enhancing GLUT4 and reducing insulin receptor (InR) expression, independent of insulin expression.

In conclusion, rutin ameliorates the progression of diabetes and associated neurological complications. Such central mechanistic viewpoint could be exploited as a new target for therapeutic management of diabetes.

INTRODUCTION

Diabetes mellitus (DM) is a complex metabolic disorder characterized by chronic, prolonged and consistent hyperglycemia. Diabetes develops when the pancreas is no longer able to produce insulin, or when the body cells are inefficient in utilizing the insulin, based on which diabetes is classified as type 1 and type 2 respectively. Insulin is a hormone synthesized by the β cells of pancreas that acts like a key to let glucose from the blood into the cell to produce energy. Type 1 diabetes mellitus (T1DM) is characterized by pancreas failure to produce insulin, therefore also known as Insulin dependent diabetes mellitus (IDDM). On the other hand, type 2 diabetes mellitus (T2DM) is characterized by Insulin resistance (IR), a condition in which the ability of body cells to respond to insulin is compromised. This condition is also known as Non-insulin dependent diabetes mellitus (NIDDM). Some common symptoms of diabetes are polydipsia, polyuria, polyphagia, weight loss, fatigue and numbness in the extremities. Diabetes leads to development of secondary complications like cardiac arrest, stroke, renal failure, amputation, blindness and neuropathy, increasing the probability of premature death [1]. According to the 7th edition of International Diabetes Federation Atlas [2], diabetes affects approximately 415 million adults (1 in 11); expected to rise to 642 million (1 in 10) by 2040. Diabetes caused 5 million deaths (1 in every 6 seconds), and more than 20.9 million live births (1 in 7 births) were affected during pregnancy in 2015. 46% (1 in 2) people with diabetes remain undiagnosed, and diabetes caused at least USD 673 billion dollars in health expenditure in 2015. Approximately, 90% of the diabetes cases belong to T2DM category.

Apart from the most common and widely studied peripheral diabetic complication, such as cardiovascular problems, neuropathy, nephropathy, and retinopathy, there exists a significant CNS pathology too. T2DM causes excessive oxidative stress leading to various structural and functional abnormalities in the brain, including neurodegeneration, dementia, blood brain barrier (BBB) disruption, organelle dysfunctioning (mitochondria, endoplasmic reticulum, microtubule and chromatin) etc [3].

Since glucose is the primary fuel for brain, insulin seems to be an integral part of its homeostasis. But, for decades it was considered that glucose uptake in brain is independent of insulin [4-6], which could be brain's innate defense mechanism against hyperglycemia. This notion has however been challenged by studies that reveal a more intricate role of insulin in the brain [7-9]. Additionally, new findings suggest a high concentration of insulin receptors (InR's) in various

brain regions like hypothalamus, hippocampus, cortex and olfactory bulb i.e. areas that modulate behavior, reproduction, learning, memory and cognition [7, 10]. Insulin mediates several different brain functions like neurotransmitter reuptake [11], glycogen metabolism [12], appetite control and satiety [13-15]. Also, locally synthesized insulin in brain has been reported [16, 17].

Central prominence of diabetic complications can be judged by the fact that Alzheimer's is now being hypothesized to be a type 3 form of diabetes [Reviewed in [18]].

Hippocampus, a brain region involved in learning and memory functions, and the prominent site for adult neurogenesis, is rich in InR [19-21]. InR activation leads to synthesis and translocation of glucose transporter 4 (GLUT4) necessary for glucose uptake and hence maintaining energy homeostasis of the neuron. Further, studies suggest that diabetes is associated with reduced hippocampal neurogenesis [22-25]. Additionally, insulin resistance (IR) developed during T2DM lowers glucose uptake, causing neuronal starvation and hence degeneration which ultimately translates to impaired neurobehavioral outcomes [26, 27]. Interestingly, Amyloid-β (Aβ), is cleared by insulin degrading enzyme (IDE), which means that during diabetes impaired insulin signaling could be somehow associated with hippocampal accumulation of Aβ plaques leading to development of Alzheimer's [28, 29]. This indicates that there exists a delicate relationship between diabetes, brain and central glucose homeostasis, a critical research gap we address in our study. We suggest that, diabetes is a ticking time bomb for development of catastrophic neurological complications. This progression is slow and hence remains unnoticed and to this date under studied. Since diabetes has always been regarded as the disease of periphery, all of the current medication targets peripheral glucose clearance and there is literally no consideration of the central aspects, which is why despite the glycemic control, neurological complications keep on progressing day by day from where there is no turning back. Importance of central aspects of glucose management can be appreciated from the fact that although being about 2% of the body weight, brain utilizes approximately 60-70% of total body glucose [30]. So the research gap we address in our study is to understand the central control over glucose homeostasis via insulin signaling and how it may interfere with disease progression. Diabetes to this date remains non treatable and irreversible, and hence CNS targeted therapeutics might be a new and exciting research avenue in near future.

The second part of our study comprises of the neurological complications of stress induced depression and how it triggers the switch to diabetes. Stress is a body's innate defense

mechanism against day to day troubles. However, chronic and prolonged stress stems devastating effects on the body, especially brain. Chronic stress is the direct causative agent of neurological complications like neurodegeneration, reduced synaptic plasticity, and behavioral abnormalities like depression, anxiety, cognitive decline, dementia etc [31, 32]. Additionally, chronic stress reduces insulin sensitivity and mediates IR (T2DM) [33]. Insulin responsive GLUT4 controls the hippocampal glucose uptake [34] and is involved in learning & memory processes [35-37]. Further, InR's in hippocampus and cortex are essential for neuronal plasticity and cognitive functioning. IR, such as observed during depression in this study, can thus reduce synaptic plasticity and alter neurobehavioral outcomes leading to complications like Alzheimer's disease [7]. Keeping these roles of insulin signaling in mind, we aimed to evaluate InR and its responsive glucose transporter (GLUT4) in the hippocampus during chronic stress-induced neurological complications.

Previous reports from our lab had demonstrated that hydroalcoholic extract of *Urtica dioica* improved glucose intolerance and cognitive dysfunction by modulating insulin signaling in the hippocampus of both chronically stressed [38] and diabetic mice [37]. HPLC analysis had revealed the presence of high amount of rutin in *U. dioica* extract [39]. So, the rationale of our study was to determine if rutin is the principal neuroprotective component of *Urtica dioica*. None of the current therapeutics targets the central insulin signaling pathway, which forms the base of the novelty of our work. We aim to identify the role of insulin signaling in brain during chronic diabetes and the effect of rutin in it. Based on the above mentioned literature, we divided our work broadly into two objectives discussed hereafter.

OBJECTIVES

1. To evaluate the effect of rutin on diabetes and associated neurological complications using streptozotocin induced diabetes model.
2. To evaluate the effect of rutin on chronic unpredictable stress-induced depression as a risk factor for diabetes and associated neurological complications.

OBJECTIVE 1

To evaluate the effect of rutin on diabetes and associated neurological complications using streptozotocin induced diabetes model.

1. Experimental design: Adult male Swiss albino mice (5-6 months old) weighing 20–25 g were housed in the Animal House of Jaypee University of Information Technology, Waknaghat, under a 12 h light/dark cycle at 23 ± 2°C. All the experiments were performed with prior approval from Institute Animal Ethics Committee (IAEC) as per guidelines of CPCSEA. 25 animals were injected (i.p) with 50 mg/kg Streptozotocin (STZ) (in ice cold citrate buffer, pH 4.5) for 5 consecutive days between 7 and 8 am daily [40]. Diabetes developed between 5-7 days of last STZ injection. Animals with 12 h fasting glucose levels ≥ 200 mg/dl were selected for the study and divided into two groups (n=8-10) viz group 3: STZ + Vehicle (0.3% carboxymethyl cellulose and group 4: STZ + Rutin (STZ + R). 15 animals were injected (i.p) with equivalent ice cold citrate buffer (pH 4.5) for 5 consecutive days and were divided into two subgroups (n = 8-10), Group 1: Control + Vehicle (Control) and Group 2: Control + Rutin (Control + R). All the treatments including rutin (100 mg/kg) were given orally, once daily between 5-6 pm, for 8 weeks.

2. General Parameters/Biochemical Analysis: Diabetic progression was tracked by various physiological parameters like body weight, feed intake and water intake and biochemical parameters like, fasting blood glucose (FBG), oral glucose tolerance test (OGTT) and plasma insulin.

Our results showed a significant ($p < 0.001$) decline in body weight along with increased ($p < 0.001$) feed and water intake (polyphagia and polydipsia) in diabetic animals. FBG of STZ group was significantly ($p < 0.001$) higher than control group. OGTT result showed blood glucose of STZ group significantly ($p < 0.001$) higher at each time point compared to control. Rutin treatment significantly improved upon all of the abovementioned abnormalities. Rutin prevented body weight decline ($p < 0.001$), and reduced feed & water intake ($p < 0.001$). Rutin treated animals showed significantly ($p < 0.001$) reduced glycemic levels and showed the minimum area under OGTT curve ($p < 0.001$), indicating potential anti-diabetic effect of rutin. Diabetes led to a significant ($p < 0.01$) decline in serum insulin levels which was normalized by rutin treatment ($p < 0.05$).

3. Behavioral Analysis: Animals were subjected to a wide array of tests for behavioral profiling like locomotion & muscle coordination, anxiety, depression, and learning & memory.

3.1 Locomotion & muscle coordination: Locomotor activity was assessed via the total number of line crossings of an open field. Diabetes led to a significant ($p < 0.001$) decline in locomotion, which was improved by rutin treatment ($p < 0.001$). Muscle coordination was assessed by beam walk test. STZ group took significantly ($p < 0.001$) higher time to cross a 6 mm beam while rutin treated animals [both control ($p < 0.05$) and CUS ($p < 0.001$)] showed excellent muscle coordination. Our results conclude that diabetes impairs locomotion and muscle coordination. Treating animals with rutin rescues these deficits as animal show improved locomotion in the open field test, and excellent muscle coordination in beam walk test.

3.2 Anxiety: Anxiety was analyzed by Elevated plus maze (EPM) and open field test (OFT). Diabetes led to severe anxiety ($p < 0.001$), as evident by reduced time spent exploring open arm of EPM and reduced entries and time spent in the center of OFT ($p < 0.001$). Rutin treatment significantly ($p < 0.001$) reversed the diabetes induced anxiety in both EPM and OFT.

3.3 Depression: Depressive behavior was analyzed using tail suspension test (TST), forced swim test (FST) and sucrose preference test (SPT). According to TST, our results show that diabetes induced severe depression as evident by increased immobility time ($p < 0.001$) and reduced upward turns ($p < 0.001$). Similar results were observed in FST, where diabetes led to significantly ($p < 0.001$) higher immobility time compared to control. In case of SPT, diabetes induced anhedonia as evident by decreased preference for sucrose ($p < 0.01$). Rutin showed potent antidepressant activity by reducing immobility ($p < 0.001$) in TST and FST, and increasing sucrose preference ($p < 0.001$).

3.4 Learning & Memory: Cognitive functioning was assessed via **l**earning & memory abilities, was the most extensively studied parameter of all. Passive avoidance-step through (PA-ST), passive avoidance-step down (PA-SD), novel object recognition (NOR) and morris water maze (MWM) were the test methods used. In case of PA-ST and PA-SD, diabetes led to severe decline in memory as the animals were unable to retain their memory of aversive stimuli on day 1 ($p < 0.001$) and day 7 ($p < 0.001$). Rutin treatment prevented this damage and animals had intact memory for aversive stimuli even on day 7. In case of NOR, diabetic animals were unable to differentiate between a novel and a familiar interaction ($p < 0.001$). Rutin treatment ($p < 0.001$)

animals had intact novelty preference profile since they remembered a previous encounter (familiarity). Diabetes also led to a significant ($p < 0.001$) decline in spatial memory as animals were unable to locate the platform in MWM test. Rutin treated animals showed excellent spatial memory as evident by increased number of platform crossings ($p < 0.001$) and time spent around the platform ($p < 0.001$). So to conclude, diabetes caused significant cognitive decline and had devastating effects on learning & memory ability, which was not only prevented but improved upon by rutin as compared to vehicle treated diabetic and control animals.

4. Neurodegeneration: Golgi-cox method was used to study the CA3 hippocampal cytoarchitecture and neuronal morphology. Diabetes led to a significant ($p < 0.001$) reduction in number of intersections, primary branches, and spike density. Rutin treatment alleviated this damage and the neurons had significantly ($p < 0.001$) higher intersections, primary branches, and spike density.

5. Proteomics: Hippocampal **e**xpression analysis of insulin signaling proteins viz insulin, insulin receptor (InR) and glucose transporter-4 (GLUT4) was performed to get better insight into the effect of diabetes and rutin treatment through western blot and immunofluorescence studies.

Diabetes resulted in reduced expression of insulin ($p < 0.01$), but InR and GLUT4 expression didn't change significantly ($p > 0.05$). Rutin treatment significantly upregulated the insulin signaling by increasing expression of insulin ($p < 0.001$), InR ($p < 0.001$) and GLUT4 ($p < 0.001$). Similar results were obtained by immunofluorescence study of InR and GLUT4.

These results suggest that although diabetic brain might be independent of insulin signaling, but its upregulation (as done by rutin) resulted in improving the diabetic parameters and complications.

6. Conclusion: Long standing diabetes induces behavioral dysfunction which was associated with neurodegeneration, reduced synaptic plasticity and reduced insulin expression in the hippocampus. Rutin treatment alleviated hyperglycemia and improved behavioral functioning, improve synaptic plasticity and neuronal survival. Rutin seems to be acting through insulin signaling by upregulating insulin, InR and GLUT4, and therefore might find a beneficial role in management of IR and T2DM.

OBJECTIVE 2

To evaluate the effect of rutin on chronic unpredictable stress-induced depression as a risk factor for diabetes and associated neurological complications.

1. Experimental Design: Adult male Swiss albino mice (5-6 months old) weighing 20–25 g were housed in the Animal House of Jaypee University of Information Technology, Waknaghat, under a 12 h light/dark cycle at 23 ± 2°C. Animals were randomly divided into four groups ($n \geq 8$); Group-1: Control, Group-2: Control + rutin (Control + R), Group-3: Chronic Unpredictable Stress (CUS) and Group-4: CUS + rutin (CUS + R). Control and CUS group received vehicle (0.3% carboxymethyl cellulose). Rutin treatment groups received 100 mg/kg rutin (suspended in the vehicle). All the treatments were given orally daily between 7 and 8 am for 21 days. CUS groups received unpredicted stress for 21 days randomly between 8 am and 6 pm and were kept in an isolated room to avoid any sort of contact with control animals.

1.1 Chronic Unpredictable Stress Procedure: Animals were subjected to a 21-day CUS paradigm as described previously with slight modifications [38]. Behavioral tests were performed between day 21–25, after which animals were sacrificed and samples were processed for further studies.

2. Biochemical Analysis: Body weight, food & water intake, FBG, OGTT, serum insulin levels, HOMA-IR index and cortisol levels were analyzed to understand the metabolic condition during CUS. Symptomatically, CUS resulted in a significant ($p < 0.001$) reduction in body weight, even though the changes in water intake were insignificant ($p > 0.05$) compared to control group. Interestingly, rutin treated animals (both control and CUS) showed increased feed intake ($p < 0.001$).

CUS led to development of pre-diabetes with significantly ($p < 0.001$) increased FBG. Serum insulin and cortisol levels were increased too ($p < 0.001$). IR was revealed as evident by increased ($p < 0.001$) HOMA-IR index and impaired OGTT as blood glucose at each time point and area under OGTT curve in CUS remained significantly higher ($p < 0.001$), compared to control. Rutin treatment significantly ($p < 0.001$) improved the FBG and OGTT profile. Additionally, CUS resulted in a significantly ($p < 0.001$) increased in serum cortisol levels, which were significantly reduced by rutin treatment ($p < 0.01$).

3. Behavioral Analysis: Animals were subjected to a wide array of tests for behavioral profiling like locomotion & muscle coordination, anxiety, depression, and learning & memory.

3.1 Locomotion & muscle coordination: Locomotor activity was assessed via the total number of line crossings of an actophotometer and open field. According to actophotometer analysis, neither CUS and nor treatment caused any significant change in locomotion. Although on the basis of open field test, CUS led to a significant ($p < 0.001$) decline in locomotion, which was improved by rutin treatment ($p < 0.001$).

Muscle coordination was assessed by beam walk test. CUS subjected animals took significantly ($p < 0.05$) higher time to cross a 6 mm beam while rutin treated animals (both control and CUS) showed excellent muscle coordination ($p < 0.001$). Our results conclude that chronic stress disrupts locomotion and muscle coordination. Treating animals with rutin rescues these deficits as animal show improved locomotion in the open field test, and excellent muscle coordination in beam walk test.

3.2 Anxiety: Anxiety was analyzed by EPM and OFT. According to EPM, CUS led to severe anxiety ($p < 0.001$), which was improved by rutin treatment ($p < 0.05$). Similarly, on the basis of number of center entries and time spent in center of OFT, rutin treatment significantly ($p < 0.001$) reversed the CUS induced anxiety ($p < 0.001$). Overall, chronic stress induced anxiety-like behavior in mice, and rutin treatment produced anxiolytic effect.

3.3 Depression: Depressive behavior was analyzed using TST, FST and SPT. According to TST, our results show that CUS induced depression as evident by increased immobility time ($p < 0.05$) and reduced upward turns ($p < 0.01$). Similar results were observed in FST, where CUS led to significantly ($p < 0.001$) higher immobility time. In case of SPT, CUS induced anhedonia as evident by decreased preference for sucrose ($p < 0.01$). Rutin showed potent antidepressant activity by reducing immobility and increasing sucrose preference ($p < 0.001$).

3.4 Learning & Memory: Cognitive functioning was assessed via **l**earning & memory abilities, was the most extensively studied parameter of all. PA-ST, PA-SD, NOR and MWM were the test methods used. In case of PA-ST and PA-SD, stress led to severe decline in memory as the CUS animals were unable to retain their memory of aversive stimuli on day 1 ($p < 0.001$) and day 7 ($p < 0.001$). Rutin treatment prevented this damage and animals had intact memory for aversive stimuli even on day 7 ($p < 0.001$). Similar results were obtained with NOR as CUS led to

memory deficits ($p < 0.001$) due to which animals were unable to differentiate between a novel and a familiar interaction. Rutin treatment prevented any such damage ($p < 0.001$) and hence animals had intact novelty preference profile since they remembered a previous encounter (familiarity). CUS also led to a significant ($p < 0.001$) decline in spatial memory as animals were unable to locate the platform in MWM test. Rutin treated animals showed excellent spatial memory as evident by increased number of platform crossings ($p < 0.001$) and time spent around the platform ($p < 0.001$). So to conclude, CUS led to significant cognitive decline and had devastating effects on learning & memory ability, which was not only prevented but improved upon by rutin as compared to vehicle treated CUS and control animals.

4. Neurodegeneration: Integrity of the hippocampal neurons was determined by hematoxylin & eosin staining of the 5 μm thick coronal hippocampal section. Neuronal damage was evaluated in CA1, CA2 and CA3 region of the hippocampus in terms of the number of viable neurons observed in one field of microscope (400 X magnification). CUS caused severe damage in the hippocampus, especially in the CA3 region, as evident by a significant ($p < 0.001$) reduction in cell number. Rutin treatment prevented ($p < 0.001$) this stress-mediated hippocampal neuronal loss and the neurons resembled normal morphology.

5. Proteomics: Hippocampal **e**xpression analysis of insulin signaling proteins viz insulin, InR and GLUT4 was performed to get better insight into the effect of CUS and rutin treatment through western blot. Insulin expression was similar in all groups and neither rutin nor CUS caused any change ($p > 0.05$). But, CUS led to a significantly increased ($p < 0.01$) expression of InR with concomitant decrease ($p < 0.01$) in GLUT4, indicating IR. Rutin treatment normalized this condition by reducing ($p < 0.05$) InR and increasing GLUT4 expression ($p < 0.001$), thereby increasing insulin sensitivity. These results were reinforced by immunofluorescence analysis of InR and GLUT4. Our results indicate that the neuromodulatory effects of rutin could be attributed to its ability to modulate insulin signaling.

6. Conclusion: In a nutshell, CUS-induced anxiety, depression and cognitive decline, which was underlined by impaired glucose homeostasis, IR (type-2-diabetes-like state), pre-diabetes and disrupted hippocampal insulin signaling. These findings suggest that intact insulin signaling machinery is essential for glucose uptake and hippocampal functioning. Herein, we propose a

novel role of rutin, which is overcoming IR. These results suggest that rutin can be used in managing IR and T2DM.

SUMMARY

To summarize, both diabetes and depression (chronic unpredictable stress) induced behavioral complications and impaired glycemic control. These complications were associated with hippocampal neurodegeneration and impaired hippocampal insulin signaling. Rutin improved the glycemic control, prevented neurodegeneration and improved the neurological outcomes by modulating insulin signaling.

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